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The N-terminal domain of αB-crystallin is protected from proteolysis by bound substrate

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Abstract

α-Crystallin, a major structural protein of the lens can also function as a molecular chaperone by binding to unfolding substrate proteins. We have used a combination of limited proteolysis at low temperature, and mass spectrometry to identify the regions of α-crystallin directly involved in binding to the structurally compromised substrate, reduced α-lactalbumin. In the presence of trypsin, α-crystallin which had been pre-incubated with substrate showed markedly reduced proteolysis at the C-terminus compared with a control, indicating that the bound substrate restricted access of trypsin to R157, the main cleavage site. Chymotrypsin was able to cleave at residues in both the N- and C-terminal domains. In the presence of substrate, α-crystallin showed markedly reduced proteolysis at four sites in the N-terminal domain when compared with the control. Minor differences in cleavage were observed within the C-terminal domain suggesting that the N-terminal region of α-crystallin contains the major substrate interaction sites.

Keywords: Mass spectrometry, limited proteolysis, chaperone, structure, α-crystallin

Introduction

α-Crystallin is both a structural protein of the mammalian lens as well as a member of the small heat shock family of chaperone proteins[1, 2]. It performs an important role in the lens by preventing the aggregation of structurally compromised proteins, thereby maintaining lens clarity[3]. This is a crucial function in the ageing lens as the extraordinary longevity of this organ leaves the resident proteins susceptible to a wide variety, and high level of potentially destabilizing modifications[4-10]. Although the chaperone function of α-crystallin has been known for ~15 years and has been studied extensively *in vitro*, there has been limited work focused on characterization of the chaperone:substrate complex itself. The reasons for this are reasonably obvious when one considers the inherent polydispersity of α-crystallin[11], combined with its ability to bind very large numbers of substrate proteins[12].

A number of studies have attempted to identify the structural region(s) of α-crystallin which are responsible for its chaperone action, with particular emphasis on regions which can bind hydrophobic molecules [13-15]. These studies present a convincing case for the identification of a hydrophobic binding site in αB-crystallin encompassing residues 75-103, and a (hydrophobic) melittin binding site at residues 70-78, and that blocking of these sites reduces the chaperone activity of α-crystallin towards thermally denatured alcohol dehydrogenase (ADH). Earlier work by these researchers also examined the ADH binding sites using chemical crosslinking, and reported residues 57-69 and 93-107 as chaperone binding regions for αB-crystallin. Although this work was carefully executed, there is a possibility that the organic crosslinkers used in the study, with which ADH was pre-labelled, were in fact the moieties binding to αB-crystallin, rather than the unfolding ADH itself.

We have chosen to approach the study of the αB-crystallin chaperone:substrate interaction using a combination of limited proteolysis and mass spectrometry (MS). Limited proteolysis is a powerful tool for probing the higher order structure of proteins, as it provides information on the surface accessibility of an enzymes target residues[16]. If peptide bonds can be shown to readily cleave in the absence of a substrate, yet exhibit restricted cleavage in the presence of substrate, it is safe to assume that these bonds are involved in a form of interaction with the substrate. In summary, limited proteolysis can provide a picture of the regions of tertiary structure involved in substrate interaction.

Earlier studies have employed limited proteolysis to examine the quaternary structure of bovine α-crystallin[17], and more recently, the relationship between chaperone activity and oligomeric size[18]. In both cases gel electrophoresis was used to monitor the extent of proteolysis with time. Although useful in visualizing truncated protein chains, electrophoresis has the disadvantages of detection limits associated with staining, very imprecise estimates of molecular weight, and the inability to monitor the peptides released by the enzymes.

In the present study we have employed nanoelectrospray-ionization MS (nanoESI-MS) to detect both the truncated αB-crystallin polypeptide chain, as well as the corresponding peptides released during digestion. This approach has allowed us to accurately determine the sites of cleavage of αB-crystallin in both the absence and presence of a reduction denatured substrate. The results suggest that the N-terminal domain of αB-crystallin is the major region involved in interaction with a model destabilized substrate protein.

Materials and Methods

Materials: All materials were purchased from Sigma-Aldrich (St. Louis, USA). Solvents were of the highest grade commercially available and obtained from Ajax Finechem (Seven Hills, Australia). MilliQ^{TM} water (Millipore, Molsheim, France) was used in all experiments.

Protein expression and purification: αB-Crystallin in the vector pET24d(+) (Novagen, U.S.A.), was expressed in the BL 21 (DE3) strain of *E. coli*. Cells were pelleted by centrifugation at 8000*g* for 10 minutes and the pellet was washed with fresh LB medium. The bacteria were disrupted at 37°C for 15 minutes with lysis buffer (50 mM Tris, 100 mM NaCl, 1mM EDTA, pH 8.0) containing 100 ug/ul of lysozyme. The protein was purified as described previously[19].

To allow compatibility for nanoESI-MS analysis, αB-crystallin was exchanged into 200 mM ammonium acetate (NH_4OAc), pH 7.0, using Millipore Biomax centrifugal filters (Bedford, USA). The concentration of αB-crystallin was determined at 280 nm using an extinction co-efficient of 0.85 $mg^{-1}mL^{-1}cm^{-1}$. The final solution was adjusted to a concentration of 5 mg/mL.

Light scattering of reduced α-lactalbumin: Aggregation of DTT-reduced α-lactalbumin was monitored using a Spectramax Plus microplate reader (Molecular Devices, CA, U.S.A.). Solutions containing 1.05 mg/mL α-lactalbumin, 25 mM EDTA, 20 mM DTT and 0, 0.5 or 1 mg/mL αB-crystallin were prepared in 200 μL of 200 mM NH_4OAc, pH 7.0. These solutions were incubated at 37°C for 60 minutes and monitored for aggregation due to the apparent absorbance at 360 nm as a result of light scattering.

Limited proteolysis: For limited proteolysis experiments, solutions containing 1.05 mg/mL α-lactalbumin, 25 mM EDTA and 0.5 mg/mL αB-crystallin in 200 mM NH_4OAc, pH 7.0 were prepared. Following addition of 20 mM DTT, solutions were heated in a thermostat controlled water bath at 37°C for 12.5 minutes. These were immediately placed on ice for 1 minute before

equilibrating at 10°C for 2 minutes. Digestion was initiated by application of either 1:25 or 1:10 (w:w) ratios of trypsin or chymotrypsin to αB-crystallin respectively. Samples were kept at 10°C while digestion proceeded. Control samples had no DTT added but were heated at 37°C in the presence of non-reduced α-lactalbumin. Digestion was quenched at various time points by taking 3 μL aliquots of the digesting solution and adding it to 3 μL of a 10 μM GluFib solution in 90% acetonitrile and 1% formic acid. These solutions were kept on ice prior to analysis by nanoESI-MS.

Mass Spectrometry: All mass spectra were acquired on a Waters Q-ToF Ultima™ (Wyntheshawe, UK) mass spectrometer using a nanoESI source. Instrument conditions included a capillary potential of 1.5 kV, cone: 40 V, rf lens1: 60 V, collision cell: 4 V, transport and aperture: 5 V and an MCP of 2100 V. For MS/MS experiments the collision cell energy was increased to between 12 and 30 V. Typically, 2 μl of solution were electrosprayed from gold-coated glass capillaries prepared in-house, and 20 scans were acquired for each MS or MS/MS spectrum. Peptide identification was carried out by *de novo* sequencing and using the Biolynx™ Protein/Peptide Editor. All spectra were processed using MassLynx™ software with minimal smoothing applied to the raw data.

Results and discussion

It is well established that α-crystallin can function as a molecular chaperone, and that this chaperone action involves hydrophobic interactions with, and binding to a substrate[19-21]. We exploited this behavior using recombinant human αB-crystallin (αB) under carefully controlled conditions in order to capture the soluble chaperone-substrate (CS) complex for further analysis. Initial experiments utilized reduced α-lactalbumin (RAL) as the substrate in a commonly performed heat reduction assay, where the absorbance at 360 nm of RAL was measured over time in the absence and presence of αB (Fig.1). Without αB, RAL was observed to undergo a rapid increase in absorbance after 8 minutes, indicative of an aggregation process. This aggregation was mitigated to an extent by the addition of αB at a ratio of 0.5:1 w/w, as evidenced by a delay in the onset of aggregation and a change in the shape of the absorbance curve. When present at 1:1 w/w, the chaperone efficacy of αB was markedly augmented. We chose to isolate a CS complex from the reaction containing 0.5:1 w/w αB:RAL as this was expected to contain species where the primary substrate binding sites of αB were occupied by RAL. Thus for all subsequent limited proteolysis and mass spectrometry experiments, a solution containing 0.5:1 w/w αB:RAL was heated for 12.5 minutes at 37°C prior to immersion in an ice bath, in order to preserve the integrity of the CS complex.

Initial limited proteolysis experiments were performed using the αB:RAL complexes and trypsin as the protease. In order to accurately map the sites of substrate binding it was important to minimize the kinetic activity of the system during proteolysis. For this reason, complexes were digested for 60 minutes at 10°C as it has previously been shown that subunit exchange of αB is negligible at this temperature[22]. Samples were removed at intervals during the digestion process enabling us to monitor the sites and extent of proteolysis with time. Figure 2 shows the

spectra transformed to a mass scale of αB digested with trypsin for the periods indicated in the absence (left panel) and presence (right panel) of RAL. Under the conditions used, it is clear that the major product resulting from tryptic cleavage was residues 1-157 of αB ($\alpha B_{1\text{-}157}$), indicating that cleavage at R157 dominated.

In the absence of RAL, the amount of $\alpha B_{1\text{-}157}$ was observed to increase rapidly over the period of digestion, occurring at greater abundance than the full length αB after 60 minutes (left panel, bottom spectrum). A minor cleavage product corresponding to $\alpha B_{1\text{-}163}$ was also observed. The peak at ~19750 Da is a truncation product ($\alpha B_{1\text{-}171}$) from the *E. coli* expression system which co-purified with the full length αB. When αB was digested after incubation in the presence of RAL, a similar spectrum was observed, however the intensity of the peak arising from $\alpha B_{1\text{-}157}$ was significantly lower at all time points. After 60 minutes $\alpha B_{1\text{-}157}$ accounted for only 26% of the combined intensities of $\alpha B_{1\text{-}157}$ and $\alpha B_{1\text{-}175}$. This is in sharp contrast to digestion in the absence of RAL, where $\alpha B_{1\text{-}157}$ accounted for 53% of the combined intensities, and suggests that the region of sequence around R163 is involved in interactions with an unfolding substrate. It is also evident from this data that trypsin has very little access to both the N-terminal and C-terminal domains under the conditions used here, considering the number of potential cleavage sites present in the sequence (Fig. 3A), thus we decided to assess the proteolytic efficacy of chymotrypsin towards αB:RAL complexes. The predicted cleavage sites for chymotrypsin are indicated in figure 3B.

An example of the raw mass spectral data obtained for the chymotryptic cleavage of αB and αB:RAL complex is shown in figure 3C. Unlike the results presented in figure 2, this time we monitored the peptides cleaved from αB, rather than the residual protein chain. The region of the

spectra presented contains peaks arising from peptides 10-47 and 18-47 of αB. Peptide 10-47 has a mass of 4501.1 Da, however it appears as a species with four positive charges at *m/z* 1126.3. After five and fifteen minutes of digestion with chymotrypsin, signal arising from this peptide is clearly more abundant in the absence of RAL (fig. 3C). Similarly, signal at *m/z* 1147.6, arising from the triply charged 18-47 peptide was also more abundant in the absence of RAL compared with the αB:RAL complex.

This series of spectra was used to identify and compare all peptides arising from either tryptic or chymotryptic digestion of a control incubation of αB and an αB:RAL complex (Fig. 4). Furthermore, in order to more accurately quantify the relative abundances of cleavage products, we decided to normalize the peak intensities of the cleaved peptides against the signal arising from a standard amount of GluFib, added to the digest mixture after quenching. The data for a trypsin digest of αB in the absence of substrate (Fig. 4A, — categories) agree with those obtained for measuring the residual protein chain (Fig. 2); namely that cleavage at R157 gave rise to the major products, peptides 158-175 and 158-163. Signal corresponding to peptide 164-175 also suggests that R163 is a minor cleavage point, again in agreement with the data in figure 2. The presence of peaks corresponding to peptides 1-11 and 12-22 implies that some access was available to trypsin to the N-terminal region of αB. When RAL formed a complex with αB, the abundance of the tryptic digest products was seen to be significantly reduced (Fig. 4A, **+** categories). Again this is in agreement with the data in figure 2, strongly suggesting that the C-terminus of αB is involved in interactions with RAL.

Chymotrypsin, which cleaves on the carboxyl side of bulky/aromatic residues, was able to cleave αB at more sites than trypsin (Fig. 4B), as evidenced by the greater number of peptides observed. Cleavage at residues W9, F17, F24 and F47 provided an important map of the N-terminal domain

(residues 1-59), whereas peptide 76-122 constitutes approximately half of the C-terminal, or α-crystallin domain (residues 67-162). In the presence of RAL, significantly lower ion intensities were observed for the N-terminal peptides compared with the control, suggesting that binding of the reduced substrate prevented access to this region by the enzyme. The fact that peptides 18-47 and 25-47 showed similarly reduced accessibility to peptide 10-47 implies that the sequence interacting with RAL is probably located on the 25-47 portion of the chain, although the observation of differences in tryptic peptides 1-11 and 12-22, suggests some additional interaction with the N-terminus.

Within the α-crystallin domain, the peptide 76-122 showed no obvious difference in intensity when the digest was performed in the presence or absence of bound RAL. On closer inspection, however, the control sample has an approximately steady state population of this peptide over the period of sampling, whereas the substrate bound sample exhibits an incremental increase. This suggests that in the case of the control, peptide 76-122 was able to be further degraded after release from the protein, whereas the RAL bound protein remained protected. This is reflected by differences in the evolution of peptide 123-175, which suggest that there is slightly greater accessibility to Y122 in the control, compared with the complexed sample. Overall, the above data suggest that the major CS interactions occurred in the N-terminal domain of the αB subunits, while less prominent interactions were observed within the C-terminal domain.

α-Crystallin is amphiphilic in structure with a hydrophobic N-terminal region and a hydrophilic C-terminal domain [23]. α-Crystallin associates via hydrophobic interactions amongst the N-terminal domains, and these interactions also mediate subunit exchange[24]. Mutation of phenylalanine residues F24 and F27 in the N-terminal domain of αB-crystallin was found to abolish chaperone activity[25], suggesting an important role for these residues either in

hydrophobic interaction with substrate, subunit exchange, or both. We have shown in the present study that access to F24 and F17 is significantly reduced in the presence of bound substrate when compared to a control sample of αB-crystallin. This finding supports the proposal that the phenylalanine-rich region of the N-terminal domain is required for chaperone-like activity[25]. The absence of a cleavage product at F27 or F28 suggests that these residues may be involved in intersubunit interactions, irrespective of the presence of substrate.

The finding that the C-terminal extensions were less prone to proteolysis in the presence of substrate may reflect the proximity of R157 to the conserved I-X-I/V motif (residues 159-161). This motif has been shown to make contacts with a hydrophobic patch in the α-crystallin domain of neighboring subunits in the absence of substrate[26], however these interactions may well have been transferred to hydrophobic regions on RAL during the chaperoning event. Such a scenario would imply a 'capture' role for the C-terminal extensions, effectively using the IXI/V sequence to reach out and adhere to exposed hydrophobic regions of substrate proteins.

Although chaperoning by α-crystallin appears to be a binary event, involving no cofactors or direct energy input, the differences in accessibility to enzyme activity could result from structural re-arrangements as well as interference binding of the substrate. For example, binding of substrate at the N-terminal domain of α-crystallin may, in an allosteric fashion, alter the nature and rate of the interactions in other regions of the oligomer. Such considerations are necessary when considering the plethora of functional studies which have been published regarding α-crystallin, including the present one. In conclusion, however, it is clear that both the N-terminal domain and the C-terminal extensions of αB-crystallin are more protected from enzymatic cleavage in the presence of bound substrate. Whether this is steric in nature, or the result of indirect structural changes is yet to be determined.

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Figure legends

Figure 1: Increase in light-scattering due the aggregation of DTT-reduced α-lactalbumin (1.05 mg/mL) in the presence (squares and triangles) or absence (diamonds) of αB-crystallin at the w/w ratios indicated. Incubations were performed at 37°C in 200 mM ammonium acetate, pH 7.0. The vertical dotted line indicates the point at which the 1:0.5 w/w RAL:αB mixture was quenched in an ice bath, prior to subsequent limited proteolysis.

Figure 2: Mass spectra of αB-crystallin and C-terminally truncated species as a result of limited tryptic digestion. Spectra were acquired for samples which were quenched at 2, 15, 30 and 60 minutes respectively. The spectra on the left correspond to a control digestion of αB-crystallin which had been incubated for 12.5 minutes at 37°C in the absence of RAL, whereas those on the right were acquired on a sample containing αB:RAL complexes. In each case the raw data have been transformed to a mass scale for clarity.

Figure 3: (A) Sequence of αB-crystallin showing the predicted cleavage sites for the enzyme trypsin. Only the sites indicated by upward arrows were observed at significant levels under the conditions used for digestion (10°C, pH 7.0). (B) Sequence of αB-crystallin showing the observed cleavage sites for the enzyme chymotrypsin. (C) Portion of the raw mass spectrum showing signal arising from two chymotryptic cleavage products of αB-crystallin: peptides 10-47 and 18-47. The spectra reveal the relative populations of the peptides after five minutes (upper spectra) and fifteen minutes (lower spectra) of digestion with chymotrypsin, in the presence or absence of RAL. Signal arising from both peptides is clearly more abundant in the absence of

RAL at both time points. The number of charges on each of the peptides is indicated by superscripts.

Figure 4: Relative abundances of peptides released from αB-crystallin over time by limited proteolysis. (A) Trypsin limited digest of αB-crystallin in the absence (—) or presence (+) of reduced α-lactalbumin. A ratio of 1:0.5 α-lactalbumin to αB (w:w) was used and samples were heated for 12.5 minutes at 37°C prior to digestion at 10°C. Error bars show standard deviations. (B) Chymotrypsin limited digest of αB-crystallin in the absence (—) or presence (+) of reduced α-lactalbumin. Conditions used were identical to those for the tryptic digests. Error bars show standard deviations.

Figure 1

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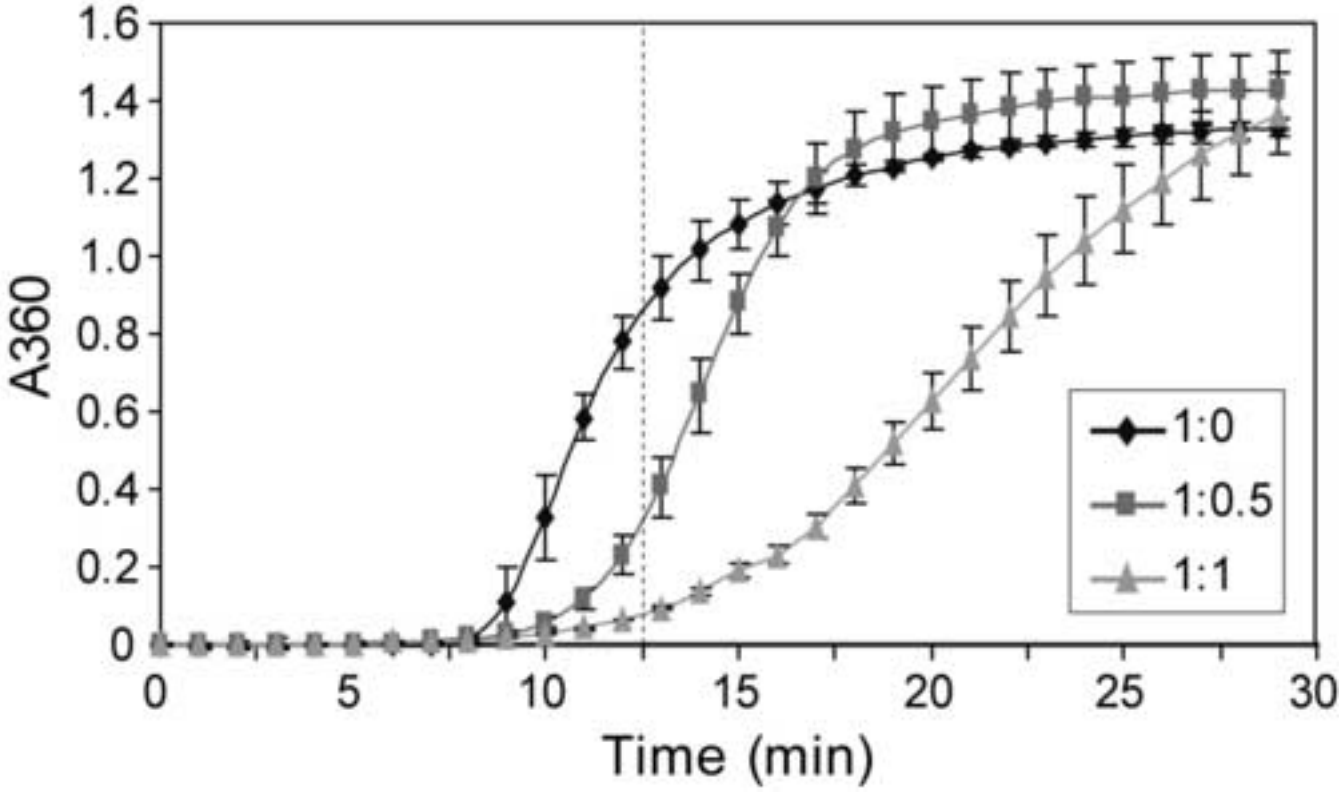

Figure 2

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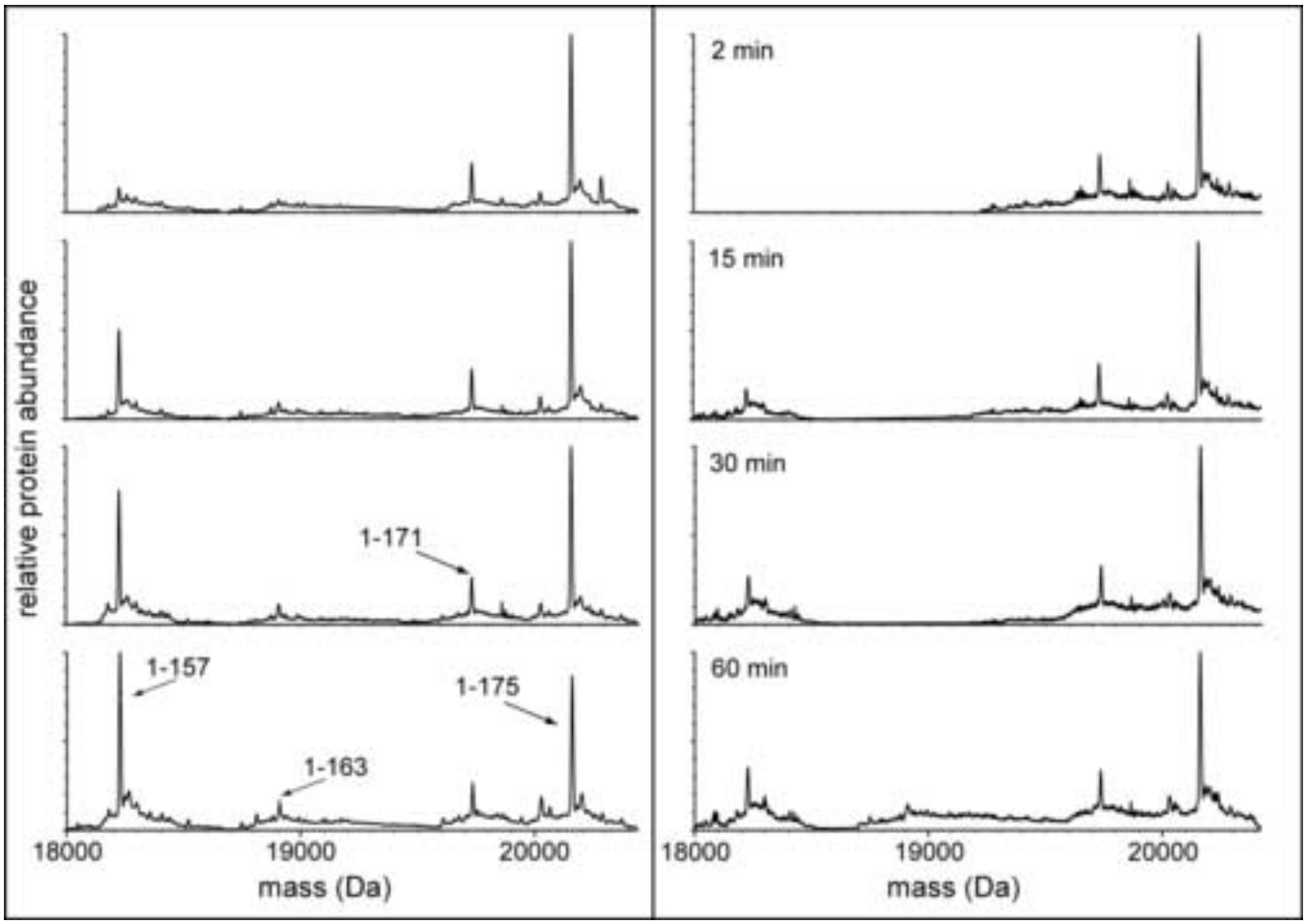

Figure 3

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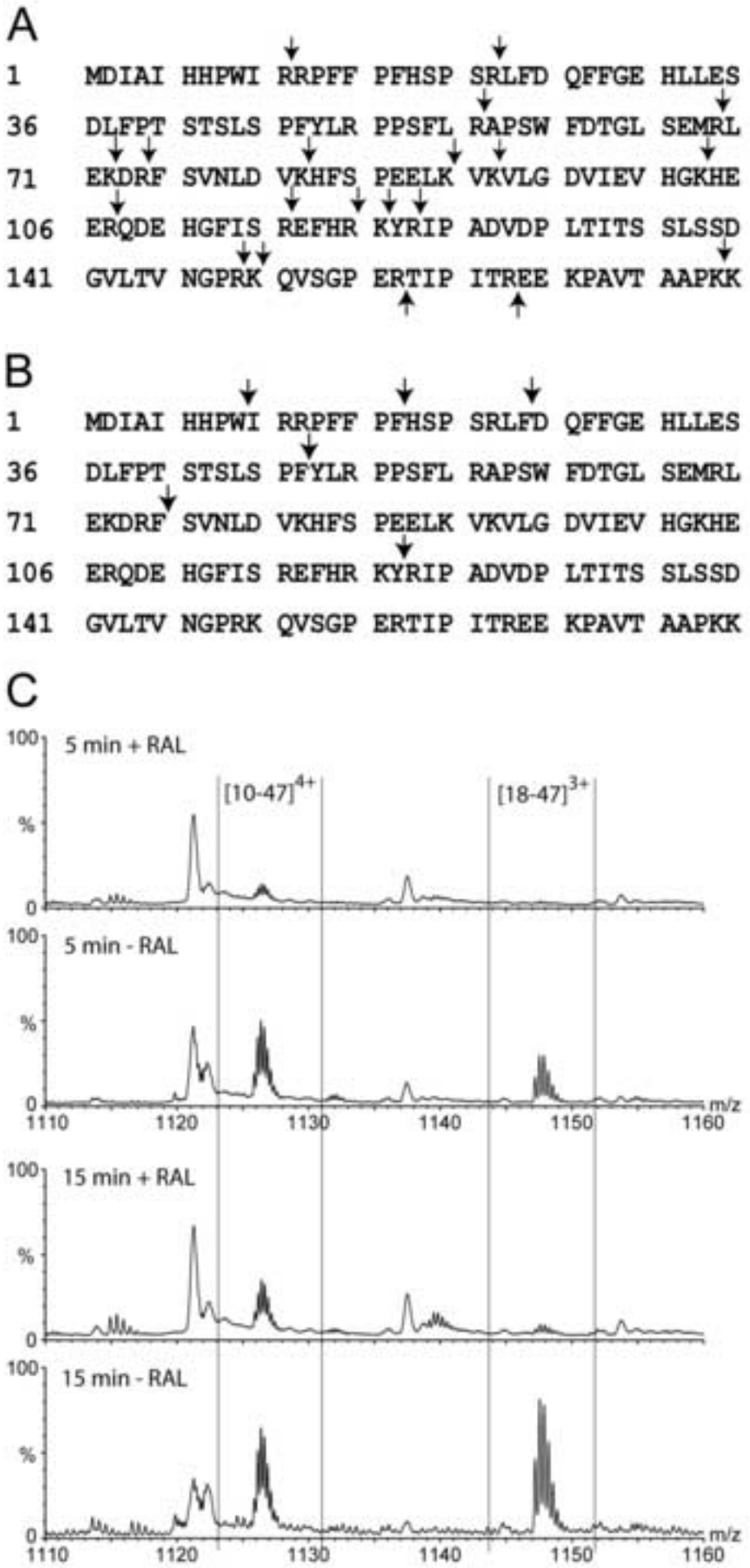

Figure 4

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